(19) Europäisches Patentamt
European Patent Offic
Offic uropéen d s br vets

(11) EP 0 489 780 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
04.11.1998 Bulletin 1998/45

(21) Application number: **90912715.1**

(22) Date of filing: **28.08.1990**

(51) Int Cl.[6]: **C12N 15/62**

(86) International application number:
PCT/US90/04840

(87) International publication number:
WO 91/03550 (21.03.1991 Gazette 1991/07)

(54) PREPARATION OF FUSION PROTEINS

HERSTELLUNG VON FUSIONSPROTEINEN

PREPARATION DE PROTEINES DE FUSION

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB IT LI LU NL SE

(30) Priority: **29.08.1989 US 399874**

(43) Date of publication of application:
17.06.1992 Bulletin 1992/25

(73) Proprietors:
- **HOECHST AKTIENGESELLSCHAFT**
  65926 Frankfurt am Main (DE)
- **THE GENERAL HOSPITAL CORPORATION**
  Boston, MA 02114 (US)

(72) Inventors:
- **STENGELIN, Siegfried**
  D-6238 Hofheim am Taunus (DE)
- **ULMER, Wolfgang**
  D-6239 Eppstein/Taunus (DE)
- **HABERMANN, Paul**
  D-6239 Eppstein/Taunus (DE)
- **UHLMANN, Eugen**
  D-6246 Glashutten/Taunus (DE)
- **SEED, Brian**
  Boston, MA 02114 (US)

(56) References cited:

EP-A- 195 680	**EP-A- 211 299**
US-A- 4 366 246	**US-A- 4 431 739**
US-A- 4 652 639	**US-A- 4 769 326**

- **Gene, Vol. 44, 1986, OLIPHANT et al. "Cloning of ramdom-sequence oligodeoxynucleotides", pages 177-183, see entire document.**
- **Proceedings of the National Academy Sciences, Vol. 86, issued April 1989, BUTT et al, "Uhiquitin fusion augments the yield of Cloned Gene products in Escherichia Coli", page 2540-2544, see entire document.**
- **Science, Vol. 234, issued 10 October 1986; BACHMAIR et al., "In Vivo Half-Life of a Protein is a Function of its Amino-Terminal Residue", pages 179-186, see entire document.**

EP 0 489 780 B1

Printed by Jouve, 75001 PARIS (FR)

Description

FIELD OF THE INVENTION

The present invention relates to fusion proteins and a process for preparing fusion proteins. The invention also pertains to various oligonucleotide and amino acid sequences which make up proteins of the present invention.

BACKGROUND OF THE INVENTION

Proteins, which in addition to the desired protein, also have an undesirable constituent or "ballast" constituent in the end product are referred to as fusion proteins. When proteins are prepared by genetic engineering, the intermediate stage of a fusion protein is utilized particularly if, in direct expression, the desired protein is decomposed relatively rapidly by host-endogenous proteases, causing reduced or entirely inadequate yields of the desired protein.

The magnitude of the ballast constituent of the fusion protein is usually selected in such a manner that an insoluble fusion protein is obtained. This insolubility not only provides the desired protection against the host-endogenous proteases but also permits easy separation from the soluble cell components. It is usually accepted that the proportion of the desired protein in the fusion protein is relatively small, i.e. that the cell produces a relatively large quantity of "ballast".

The preparation of fusion proteins with a short ballast constituent has been attempted. For example, a gene fusion was prepared which codes for a fusion protein from the first ten amino acids of β-galactosidase and somatostatin. However, it was observed that this short amino acid chain did not adequately protect the fusion protein against decomposition by the host-endogenous proteases (US-A 4 366 246, Column 15, Paragraph 2).

From EP-A 0 290 005 and 0 292 763, we know of fusion proteins, the ballast constituent of which consists of a β-galactosidase fragment with more than 250 amino acids. These fusion proteins are insoluble, but they can easily be rendered soluble with urea (EP-A 0 290 005).

Although fusion proteins have been described in the art, the generation of fusion proteins with desirable traits such as protease resistance is a laborious procedure and often results in fusion proteins that have a number of undesirable characteristics. Thus, a need exists for an efficient process for producing fusion proteins with a number of attractive traits including protease resistance, proper folding, and effective cleavage of the ballast from the desired protein.

SUMMARY OF THE INVENTION

The present invention relates to a process for the preparation of fusion proteins. Fusion proteins of the present invention contain a desired protein and a ballast constituent. The process of the present invention involves generating an oligonucleotide library (mixture) coding for ballast constituents, inserting the mixed oligonucleotide (library) into a vector so that the oligonucleotide is functionally linked to a regulatory region and to the structural gene coding for the said desired protein, and transforming host cells with the so-obtained vector population. Transformants are then selected which express a fusion protein in high yield.

The process of the present invention further includes oligonucleotide coding for an amino acid or for a group of amino acids which allows an easy cleavage of the desired protein from the said ballast constituent. The cleavage may be enzymatic or chemical.

The invention also pertains to an oligonucleotide designed so that it leads to an insoluble fusion protein which can easily be solubilized. Fusion proteins of the present invention thus fulfill the requirements established for protease resistance.

Furthermore, oligonucleotide of the present invention may be designed so that the ballast constituent does not interfere with folding of the desired protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 and its continuation in Figure Ia and Figure 1b show the construction of plasmid population (gene bank) pINT4x from the known plasmid pH154/25* via plasmid pINT40. Other constructions have not been graphically presented because they are readily apparent from the figures.

Figure 2 is a map of plasmid pUH10 containing the complete HMG CoA reductase gene.

Figures 3 and 3a show construction of pIK4, a plasmid containing the mini-proinsulin gene.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a process for the preparation of a fusion prot in characterized in that a mixed oligonucleotide is constructed which codes for the ballast constituent of the fusion protein. The oligonucleotide mixture is intro-

duced in a vector in such a manner that it is functionally linked to a regulatory region and to the structural gene for the desired protein. Appropriate host cells are transformed with the plasmid population obtained in this manner, and the clones producing a high yield of coded fusion protein are selected. Advantageous embodiments of this invention are explained below:

The oligonucleotide advantageously codes at the 3'-end an amino acid or a group of amino acids which permits or permit easy and preferably enzymatic cleavage of the ballast constituent from the desired protein. According to another implementation form, an oligonucleotide is constructed that yields an insoluble fusion protein which can easily be made soluble. In particular, an oligonucleotide is preferably constructed which codes for a ballast constituent that does not disturb the folding of the desired protein.

For practical reasons, the construction, according to the invention, of the oligonucleotide for the ballast constituent causes the latter to be very short.

It was surprising to observe that, even when they have an extremely short ballast constituent, fusion proteins not only fulfill the requirements established for protease resistance, but are also produced at a high expression rate and, if desired, the fusion protein is insoluble, can easily be rendered soluble. In the dissolved or soluble state, the short ballast constituent according to the invention then permits a sterically favorable conformation of the desired protein so that it can be properly folded and easily separated from the ballast constituent.

If the desired protein is formed in a pro-form, the ballast constituent can be constituted in such a manner that its cleavage can occur concomitantly with the transformation of the pro-protein into the mature protein. In insulin preparation, for example, the ballast constituent and the C chain can be removed simultaneously, yielding a derivative of the mature insulin which can be transformed into insulin without any side reactions involving much loss.

The short ballast constituent according to the invention is actually shorter than the usual signal sequences of proteins and does not disturb the folding of the desired protein. It therefore need not be eliminated prior to the final processing step yielding the mature protein.

The oligonucleotide coding for the ballast constituent preferably contains the DNA sequence (coding strand)

$$(DCD)_x$$

in which D stands for A, G or T and x is 4-12, preferably 4-8.

In particular, the oligonucleotide is characterized by the DNA sequence (coding strand)

$$ATG \quad (DCD)_y \quad (NNN)_z$$

in which N in the NNN triplet stands for identical or different nucleotides, excluding stop codons, z is 1-4 and y+z is 6-12, preferably 6-10, wherein y is at least 4. It has proved advantageous for the oligonucleotide to have the DNA sequence (coding strand)

$$ATG \quad (DCD)_{5-8} \quad (NNN)$$

especially if it has the DNA sequence (coding strand)

$$ATG \quad GCW \quad (DCD)_{4-8} \quad CGW$$

or, advantageously

$$ATG \quad GCA \quad (DCD)_{4-7} \quad CGW$$

in which W stands for A or T.

The above-mentioned DNA model sequences fulfill all of these requirements. Codon DCD codes for amino acids serine, threonine and alanine and therefore for a relatively hydrophilic protein chain. Stop codons are excluded and selection of the amino acids remains within manageable scope. The following is a particularly preferable embodiment of the DNA sequence for the ballast constituent, especially if the desired protein is proinsulin:

ATG GCW $(DCD)_{y'}$ ACG CGW

or

ATG GCD $(DCD)_{y'}$ ACG CGT

in which y' signifies 3 to 6, especially 4 to 6.

The second codon, GCD, codes for alanine and completes the recognition sequence for the restriction enzyme NcoI, provided that the anterior regulation sequence ends with CC. The next to last triplet codes for threonine and, together with the codon CGT for arginine, represents the recognition sequence for restriction enzyme MluI. Consequently, this oligonucleotide can be easily and unambiguously incorporated in gene constructions.

The (NNN)z group codes in the 3' position for an amino acid or a group of amino acids that permits simple, and preferably enzymatic, separation of the ballast constituent from the subsequent protein desired. It is expedient to select the nucleotides in this group in such a manner that at the 3'-end they code the cleavage site of a restriction enzyme which permits linkage of the structural gene for the desired protein. It is also advantageous for the ATG start codon and if necessary the first DCD triplet to be incorporated into the recognition sequence of a restriction enzyme so that the gene for the ballast constituent according to the invention can easily be inserted in the usual vectors.

The upper limit of z is obtained on the one hand from the desired cleavage site for (enzymatic) cleavage of the fusion protein obtained, i.e. it encompasses codons, for example, for the amino acid sequence Ile-Glu-Gly-Arg, in case cleavage is to be carried out with factor Xa. In general, the upper limit for the sum of y and z is 12, since the ballast constituent should of course be as small as possible and, above all, not interfere with the folding of the desired protein.

For reasons of expediency, bacteria or low eukaryotic cells such as yeasts are preferred as the host organism in genetic engineering processes, provided that higher organisms are not required. In these processes, the expression of the heterologous gene is regulated by a homologous regulatory region, i.e. one that is intrinsic to the host or compatible with the host cell. If a pre-peptide is expressed, it often occurs that the pre-sequence is also heterologous to the host cell. In practice, this lacking "sequence harmony" frequently results in variable and unpredictable protein yields. Since the ballast sequence according to the invention is adapted to its environment, the selection process according to the invention yields a DNA construction characterized by this "sequence harmony".

The beginning and end of the ballast constituent are set in this construction: Methionine is at the beginning, and an amino acid or a group of amino acids that permit the desired separation of the ballast constituent from the desired protein is at the end. If, for example, the desired protein is proinsulin, as NNN a triplet coding for arginine is advantageously selected as the last codon as this permits the particularly favorable simultaneous cleaving off of the ballast constituent with the removal of the C chain. Of course, the end of the ballast constituent can also be an amino acid or a group of amino acids which allows a chemical cleavage, e.g. methionine, so that cleavage is possible with cyanogen bromide or chloride.

The intermediate amino acid sequence should be as short as possible so that folding of the desired protein is not affected. Moreover, this chain should be relatively hydrophilic so that solubilization is facilitated with undissolved fusion proteins and the fusion protein remains soluble. Cysteine residues are undesirable since they can interfere with the formation of the disulfide bridges.

The DNA coding for the ballast constituent is synthesized in the form of a mixed oligonucleotide; it is incorporated in a suitable expression plasmid immediately in front of the structural gene for the desired protein and E. coli is transformed with the gene bank obtained in this manner. Appropriate gene structures can be obtained in this way by the selection of bacterial clones that produce corresponding fusion proteins.

It was previously mentioned that the cleavage sites for the restriction enzymes at the beginning and end of the nucleotide sequence coding for the ballast constituent are to be regarded as examples only. Recognition sequences that encompass starting codon ATG and in which any nucleotides that follow may include the codon for suitable amino acids are, by way of example, also those for restriction enzymes AflIII, NdeI, NlaIII, NspHI or StyI. Since in the preferred embodiment arginine is to be at the end of the ballast sequence and since there are six different codons for arginine, additional appropriate restriction enzymes can also be found here for use instead of MluI, i.e., NruI, AvrII, AflIII, ClaI or HaeII.

However, it is also advantageous to use a "polymerase chain reaction" (PCR) according to Saiki, R.K. et al., Science 239:487-491, 1988, which can dispense with the construction of specific recognition sites for restriction enzymes.

It was previously indicated that limitation to the DNA sequence (DCD)x is for reasons of expediency and that this does not rule out other codons such as, for example, those for glycine, proline, lysine, methionine or asparagine.

The most efficient embodiment of this DNA sequenc is obtained by sel ction of good producers of the fusion protein, i.e., the fusion protein containing proinsulin. This yields the most favorable combination of regulation sequence,

ballast sequence and desired protein, as a result of which unfavorable combinations of promoter, ballast sequence and structural gene are avoided and good results are obtained with minimum expenditure in terms of the above-mentioned "sequence harmony".

Surprisingly, it was observed that the genes optimized for the ballast constituent according to the invention do not always contain the triplets preferred by E. coli. It was found that for Thr, codon ACA, which is used least frequently by E. coli, actually occurs frequently in the selected sequences. If, for example, the following amino acid sequence were optimized according to the preferred codon usage (p.c.u.) by E. coli (p.c.u.: Aota, S. et al., Nucleic Acids Research 16 (supplement): r315, r316, r391, r402 (1988)), we would obtain a totally different gene structure than that obtained according to the invention (Cf.Table 1):

Ala	Thr	Thr	Ser	Thr	Ala	Thr	Thr	
GCG	ACC	ACC	AGC	ACC	GCG	ACC	ACC	p.c.u.
GCA	ACA	ACA	TCA	ACA	GCA	ACT	ACG	invention

In the case of the fusion proteins with a proinsulin constituent, the initial starting point was a ballast constituent with 10 amino acids. The DNA sequence of the best producer then served as the base for variations in this sequence, whereupon it was noted that up to 3 amino acids can be eliminated without a noticeable loss in the relative expression rate. This finding is not only surprising, since it was unexpected that such a short ballast protein would be adequate, but also very advantageous since of course the relative proportion of proinsulin in the fusion protein increases as the ballast constituent decreases.

The significance of the ballast constituent in the protein is apparent from the following comparison:
Human proinsulin contains 86 amino acids. If, for a fusion protein according to EP-A 0 290 005, we take the lower limit of 250 amino acids for the ballast constituent, the fusion protein has 336 amino acids, only about one quarter of which occur in the desired protein. By comparison, a fusion protein according to the invention with only 7 amino acids in the ballast constituent has 93 amino acids, the proinsulin constituent amounts to 92.5%. If the desired protein has many more amino acids than the proinsulin, the relationship between ballast and desired protein becomes even more favorable.

It has been mentioned on a number of occasions that as a desired protein proinsulin represents only one preferred embodiment of the invention. However, the invention also works with much larger fusion proteins for which a fusion protein with the active domain of human 3-hydroxy-3-methylglutaryl-coenzyme A-reductase (HMG) is mentioned as an example. This protein contains 461 amino acids. A gene coding for the latter is known e.g. from EP-A 292 803.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Example 1

Construction of the gene bank and selection of a clone with high expression

If not otherwise indicated, all media are prepared according to Maniatis, T.; Fritsch, E. F. and Sambrook, J.: Molecular Cloning, Cold Spring Harbor Laboratory (1982). TP medium consists of M9CA medium but with a glucose and casamino acid content of 0.4% each. If not otherwise indicated, all media contain 50 µg/ml ampicillin. Bacterial growth during fermentation is determined by measurement of the optical density of the cultures at 600 nm (OD). Percentage data refer to weight if no other data is reported.

The starting material is plasmid pH154/25* (figure 1), which is known from EP-A 0 211 299 herein incorporated by reference. This plasmid contains a fusion protein gene (D'-Proin) linked to a trp-promoter and a resistance gene for resistance against the antibiotic ampicillin (Amp). The fusion protein gene codes a fusion protein that contains a fragment of the trpD-protein from E. coli (D') and monkey proinsulin (Proin). The gene structure of the plasmid results in a polycistronic mRNA, which codes for both the fusion protein and the resistance gene product. To suppress the formation of excess resistance gene product, initially the (commercial) trp-transcription terminator sequence (trpTer) (2) is introduced between the two structural genes. To do so, the plasmid is opened with EcoRI and the protruding ends are filled in with Klenow polymerase. The resulting DNA fragment with blunt ends is linked with the terminator sequence (2)

5'AGCCCGCCTAATGAGCGGGCTTTTTTTT3'
3'TCGGGCGGATTACTCGCCCGAAAAAAAA5' (2)

which results in plasmid pINT12 (figure 1-(3)).

The starting plasmid pH154/25* contains a cleavage site for enzyme PvuI in the Amp gene, as well as a HindIII-cleavage site in the carboxyterminal area of the trpD-fragment. Both cleavage sites are therefore also contained in pINT12. By cutting the plasmid (Figure 1-(3)) with PvuI and HindIII, it is split into two fragments from which the one containing the proinsulin gene (figure 1-(4)) is isolated. Plasmid pGATTP (figure Ia-(5)), which is structured in an analogous manner to (3) but which instead of the D'-Proin gene carries a gamma-interferon gene (Ifn) containing restriction cleavage sites NcoI and HindIII, is also cut with PvuI and HindIII and the fragment (figure Ia-(6)) with the promoter region is isolated. By ligation of this fragment (6) with the fragment (4) obtained from (3), we acquire plasmid pINT40 (figure Ia-(7)). The small fragment with the remainder of the gamma-interferon gene is cut from the latter with NcoI and MluI. The large fragment (figure Ib-(8)) is ligated with mixed oligonucleotide (9)

5'CATGGCDDCDDCDDCDDCDDCDDCDA3'
3'CGHHGHHGHHGHHGHHGHHGHTGCGC5' (9)

in which D stands for A, G or T and H signifies the complementary nucleotide. This results in plasmid population (gene bank) pINT4x (figure 1b-(10)). Mixed oligonucleotides of the present invention may be obtained by techniques well known to those of skill in the art.

The mixed oligonucleotide (9) is obtained from the synthetic mixed oligonucleotide (9a)

TTCGGGTACCGHHGHHGHHGHHGHHGHHGHTGCGCAG5'
TTGCCCATGGC3' (9a)

which is filled in with Klenow polymerase and cut with MluI and NcoI.

The strain E. coli WS3110 is transformed with the plasmid population (10) and the bacteria are plated on LB agar dishes. Six of the resulting bacterial clones are tested for their ability to produce a fusion protein with an insulin constituent. For this purpose, overnight cultures of the clones are prepared in LB medium, and 100 µl aliquots of the cultures are mixed with 10.5 ml TP medium and shaken at 37°C. At OD600 = 1 the cultures are adjusted to 20 µg/ml 3-β-indolylacrylic acid (IAA), a solution of 40 mg glucose in 100 ml water is added and the preparation is shaken for another three hours at 37°C. Subsequently 6 OD equivalents of the culture are removed, the bacteria contained therein are harvested by centrifugation and resuspended in 300 µl test buffer (37.5 mM tris of pH 8.5, 7 M urea, 1% (w/v) SDS and 4% (v/v) 2-mercaptoethanol). The suspension is heated for five minutes, treated for two seconds with ultrasound to reduce viscosity and aliquots thereof are subsequently subjected to SDS-gel electrophoresis. With bacteria that produce fusion protein, we can expect a protein band with a molecular weight of 10,350 D. It is evident that one of the clones, pINT41 (Table 1), produces an appropriate protein in relatively large quantities while no such protein formation is seen with the remaining clones. An immune blot experiment with insulin-specific antibodies confirms that the protein coded by pINT41 contains an insulin constituent.

Table 1 shows the DNA and amino acid sequence of the ballast constituent for a number of plasmid constructs. In particular, table 1 illustrates the DNA and amino acid sequence of the ballast constituent in the pINT41 fusion protein.

Table 1

1	2	3	4	5	6	7	8	9	10	11	pINT
Met	Ala	Thr	Thr	Ser	Thr	Ala	Thr	Thr	---	Arg	
ATG	GCA	ACA	ACA	TCA	ACA	GCA	ACT	ACG	---	CGT	41
				Thr	Ser	Thr			---		
***	***	***	**G	A*T	T*G	A*G	**G	***	---	***	42
		Ala		Thr	Ser	Thr	Ser		---		
***	**T	G**	***	A*T	T*T	A*T	T*A	***	---	***	43
					---	Asn	Ser		---		
***	***	***	***	***	---	AAC	T*A	***	---	***	60
								---	---		
***	***	***	***	***	***	***	***	---	---	**A	67d
							---	---	---		
***	***	***	***	***	***	***	---	---	---	**A	68d
						---	---	---	---		
***	***	***	***	***	***	---	---	---	---	**A	69d,72d
						Gly	Asn	Ser	Ala		
***	***	***	***	***	***	*G*	*A*	T**	GCA	**A	90d,91d
						Lys	---	---	---		
***	***	***	***	***	***	AA*	---	---	---	**A	93d
						Pro	---	---	---		
***	***	***	***	***	***	C**	---	---	---	**A	94d
						Met	---	---	---		
***	***	***	***	***	***	ATG	---	---	---	**A	95d

```
                                        Gly --- --- ---
*** *** *** *** *** *** *G* --- --- --- **A    96d
```

Example 2

Selection of additional clones

To detect additional suitable clones, a method according to Helfman, D.M. et al. (Proc. Natl. Acad. Sci. USA 80: 31-35, 1983) is used. TP-agar dishes, the medium of which contains an additional 40 µm/ml IAA, are utilized for this purpose. Fifteen minutes before use, the agar surface of the plates is coated with a 2-mm thick TP top agar layer, a nitrocellulose filter is placed on the latter and freshly transformed cells are placed on the filter. Copies are made of the filters which have grown bacteria colonies following incubation at 37°C, and the bacteria from the original filter are lysed. To accomplish this, the filters are exposed to a chloroform atmosphere in an desiccator for 15 minutes, subsequently moved slowly for six hours at room temperature in immune buffer (50 mM tris of pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, and 3% (w/v) BSA), which contains an additional 1 µg/ml DNase I and 40 µg/ml lysozyme, and then washed twice for five minutes in washing buffer (50 mM tris of pH 7.5 and 150 mM NaCl). The filters are then incubated overnight at 3°C in immune buffer with insulin-specific antibodies, washed four times for five minutes with washing buffer, incubated for one hour in immune buffer with a protein A-horseradish peroxidase conjugate, washed again four times for five minutes with washing buffer and colonies that have bound antibodies are visualized with a color reaction. Clones pINT42 and pINT43, which also produce fairly large quantities of fusion protein, are found in this manner in 500 colonies. The DNA obtained by sequencing and the amino acid sequences derived from it have also been reproduced in Table 1.

Example 3

Preparation of plasmid pINT41d.

Between the replication origin and the trp-promoter, plasmid pINT41 contains a nonessential DNA region which is flanked by cleavage sites for enzyme Nsp(7524)1. To remove this region from the plasmid, pINT41 is cut with NSP (7524)1, and the larger of the resulting fragments is isolated and religated. This gives rise to plasmid pINT4ld, the DNA sequence of which is reproduced in Table 2.

Table 2: DNA-Sequence of Plasmid pINT41d

```
         10                  30                  50
GTGTCATGGTCGGTGATCGCCAGGGTGCCGACGCGCATCTCGACTTGCACGGTGCACCAA
         70                  90                 110
TGCTTCTGGCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCTGTGCAGGTCGTAAATCAC
        130                 150                 170
TGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACA
        190                 210                 230
TCATAACGGTTCTGGCAAATATTCTGAAATGAGCTGTTGACAATTAATCATCGAACTAGT
        250                 270                 290
TAACTAGTACGCAAGTTCACGTAAAAAGGGTATCGACCATGGCAACAACATCAACAGCAA
        310                 330                 350
CTACGCGTTTCGTGAACCAGCACCTGTGCGGCTCCCACCTAGTGGAAGCTCTCTACCTGG
        370                 390                 410
TGTGCGGGGAGCGAGGCTTCTTCTACACACCCAAGACCCGCCGGGAGGCAGAGGACCCTC
        430                 450                 470
AGGTGGGGCAGGTGGAGCTGGGCGGGGGCCCTGGCGCAGGCAGCCTGCAGCCCTTGGCGC
        490                 510                 530
TGGAGGGGTCCCTGCAGAAGCGCGGCATCGTGGAGCAGTGCTGCACCAGCATCTGCTCCC
        550                 570                 590
TCTACCAGCTGGAGAACTACTGCAACTAATAGTCGACCTTTGCTTTCATTGTCGATGATA
        610                 630                 650
AGCTGTCAAACATGAGAATTAGCCCGCCTAATGAGCGGGCTTTTTTTTAATTCTTGAAGA
        670                 690                 710
CGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCT
        730                 750                 770
TAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTC
        790                 810                 830
TAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAA
        850                 870                 890
TATTGAAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTT
        910                 930                 950
GCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCT
        970                 990                1010
GAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATC
       1030                1050                1070
CTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTA
       1090                1110                1130
TGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACAC
       1150                1170                1190
TATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGC
       1210                1230                1250
ATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAAC
       1270                1290                1310
TTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGG
       1330                1350                1370
GATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGAC
       1390                1410                1430
GAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGC
```

```
      1450                    1470                    1490
GAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTT
      1510                    1530                    1350
GCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGA
      1570                    1590                    1610
GCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCC
      1630                    1650                    1670
CGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAG
      1690                    1710                    1730
ATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCA
      1750                    1770                    1790
TATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATC
      1810                    1830                    1850
CTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCA
      1870                    1890                    1910
GACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGC
      1930                    1950                    1970
TGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTA
      1990                    2010                    2030
CCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTT
      2050                    2070                    2090
CTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTC
      2110                    2130                    2150
GCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGG
      2170                    2190                    2210
TTGGACTCAAGACGATAGTTACCGGTAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGT
      2230                    2250                    2270
GCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGC
      2290                    2310                    2330
ATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCA
      2350                    2370                    2390
GGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATA
      2410                    2430                    2450
GTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGG
      2470                    2490                    2510
GGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCT
      2530                    2550                    2570
GGCCTTTTGCTCACATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGC

TG
```

Example 4

Fermentation and processing of pINT41d-fusion protein

(i) Fermentation: A shaking culture in LB medium is prepared from E. coli W3110 transformed with pINT41d. Fifteen µl of this culture, which has an OD = 2 are then put into 15.7 1 TP medium and the suspension is fermented 16 hours at 37°C. The culture, which at this time has an OD = 13, is then adjusted to 20 µg/ml IAA, and until the end of fermentation, after another five hours, a 50% (w/v) maltose solution is continuously pumped in at a rate of 100 ml/hour. An OD = 17.5 is attained in this process. At the end, the bacteria are harvested by centrifugation.

(ii) Rupture of Cells: The cells are resuspended in 400 ml/disintegration buffer (10 mM tris of pH 8.0, 5 mM EDTA) and disrupted in a French press. The fusion protein containing insulin is subsequently concentrated by 30 minutes of centrifugation at 23,500 g and washed with disintegration buffer. This yields 134 g sediment (moist substance).

(iii) Sulfitolysis: 12.5 g sediment (moist substance) from (ii) are stirred into 125 ml of an 8 M urea solution at 35°C. After stirring for thirty minutes, the solution is adjusted to pH 9.5 with sodium hydroxide solution and reacted with 1 g sodium sulfite. After an additional thirty minutes of stirring at 35°C, 0.25 g sodium tetrathionate is added and the mixture is again stirred for thirty minutes at 35°C.

(iv) DEAE-Anion exchange chromatography: The entire batch of (iii) is diluted with 250 ml buffer A (50 mM glycine, pH 9.0) and placed on a chromatography column which contains Fractogel(R) TSK DEAE-650 (column volume 130 ml, column diameter 26 mm) equilibrated with buffer A. After washing with buffer A, the fusion protein-S-sulfonate is eluted with a salt gradient consisting of 250 ml each buffer A and buffer B (50 mM glycine of pH 9.0, 3 M urea and 1 M NaCl) at a flow rate of 3 ml/minute. The fractions containing fusion protein-S-sulfonate are then combined.

(v) Folding and enzymatic cleavage: The combined fractions from (iv) are diluted at 4°C in a volume ratio of 1 + 9 with folding buffer (50 mM glycine, pH 10.7) and per liter of the resulting dilution 410 mg ascorbic acid and 165 µl 2-mercaptoethanol are added at 4°C under gentle stirring. After correction of the pH value to pH 10.5, stirring is continued for another 4 hours at 4°C. Subsequently, solid N-(2-hydroxyethyl)-piperazine-N'-2-ethane sulfonic acid (HEPES) is added to an end concentration of 24 g per batch-liter. The mixture which now has pH 8 is digested with trypsin at 25°C. During the process, the enzyme concentration in the digestion mixture is 80 µg/l. The cleavage course is followed analytically by RP-HPLC. After two hours, digestion can be stopped by addition of 130 µg soy bean trypsin inhibitor. HPLC shows the formation of 19.8 mg di-Arg insulin from a mixture according to (iii). The identity of the cleavage product is confirmed by protein sequencing and comparative HPLC with reference substances. The di-Arg insulin can be chromatographically purified according to known methods and transformed to insulin with carboxypeptidase B.

Example 5

Construction of plasmid pINT60

Plasmid pINT60 results in an insulin precursor, the ballast sequence of which consists of only nine amino acids. For construction of this plasmid, plasmid pINT40 is cut with Nco and MluI and the resulting vector fragment is isolated. The oligonucleotide Insul5

```
TTCGGGTACCGTTGTTGTAGTTTGAGTTGCGCAG 5'
TTGCCCATGGC 3'
```

is then synthesized, filled in with Klenow polymerase and also cut with these two enzymes. The resulting DNA fragment is then ligated with the vector fragment to yield plasmid pINT60.

Table 1 shows the DNA and amino acid sequence of the ballast constituent in this fusion protein.

Example 6

Construction of plasmid pINT67d

Plasmid pINT67d is a derivative of pINT41d in which the codon of the amino acid in position nine of the ballast sequence is deleted. That is why, like pINT60, it results in an insulin precursor with a ballast sequence of nine amino acids. A method according to Ho, S.N. et al. (Gene 77:51-59, 1989) is used for its construction. For this purpose, two separate PCR's are first performed with plasmid pINT41d and the two oligonucleotide pairs

```
TIR:      5'-CTG AAA TGA GCT GTT GAC-3'
          and
DTR8:     5'-CAC AAA TCG AGT TGC TGT TGA TGT TGT-3'
          or
DTR9:     5'-ACA GCA ACT CGA TTT GTG AAC CAG CAC-3'
          and
Insul1:   5'-TCA TGT TTG ACA GCT TAT CAT-3'.
```

This produces two fragments that are partially complementary to each other and when annealed with each other code a similar insulin precursor as pINT41d in which, however, the amino acid in position nine is absent. For completion, the two fragments are combined and subjected to another PCR together with the oligonucleotides TIR and Insul1. From the DNA fragment obtained in this manner, the structural gene of the insulin precursor is liberated with Nco and Sall and purified. Plasmid pINT41d is then also cut with these two enzymes, the vector fragment is purified and subsequently ligated with the structural gene fragment from the PCR to yield plasmid pINT67d.

The nucleotide and amino acid sequences for the ballast region have been reproduced in Table 1.

Example 7

Construction of plasmid pINT68d

Like plasmid pINT67d, plasmid pINT68d is a shortened derivative of plasmid pINT4Id in which the codons of the two amino acids in positions eight and nine of the ballast sequence are deleted. It therefore results in an insulin precursor with a ballast sequence of only eight amino acids. The procedure previously described in Example 6 is used for its construction but with the two oligonucleotide pairs

```
TIR:      5'-CTG AAA TGA GCT GTT GAC-3'
          and
DTR10:    5'-CAC AAA TCG TGC TGT TGA TGT TGT TGC-3'
          or
DTR11:    5'-TCA ACA GCA CGA TTT GTG AAC CAG CAC-3'
          and
Insul1:   5'-TCA TGT TTG ACA GCT TAT CAT-3'.
```

The nucleotide and amino acid sequences for the ballast region have been reproduced in Table 1.

Example 8

Construction of plasmid pINT69d

Plasmid pINT69d is also a shortened derivative of plasmid pINT4Id in which, however, the codons of the three amino acids in positions seven, eight and nine of the ballast sequence have been deleted. It therefore results in an insulin precursor with a ballast sequence of only seven amino acids. The procedure described in Example 6 is also used for its construction but with the two oligonucleotide pairs

```
TIR:      5'-CTG AAA TGA GCT GTT GAC-3'
          and
DTR12:    5'-CAC AAA TCG TGT TGA TGT TGT TGC CAT-3'
          or
DTR13:    5'-ACA TCA ACA CGA TTT GTG AAC CAG CAC-3'
          and
Insu11:   5'-TCA TGT TTG ACA GCT TAT CAT-3'.
```

The nucleotide and amino acid sequences for the ballast region have been reproduced in Table 1.

Example 9

Construction of plasmid pINT72d

Plasmid pINT72d is a derivative of plasmid pINT69d in which the entire C-peptide gene region, with the exception of the first codon for the amino acid arginine, is deleted. Consequently, this results in a "miniproinsulin derivative" with an arginine residue instead of a C-chain. With plasmid pINT69d as a starting point, the procedure described in Example 6 is also used for its construction but with the two oligonucleotide pairs

```
TIR:      5'-CTG AAA TGA GCT GTT GAC-3'
          and
Insu28:   5'-GAT GCC GCG GGT CTT GGG TGT-3'
          or
Insu27:   5'-AAG ACC CGC GGC ATC GTG GAG-3'
          and
Insu11:   5'-TCA TGT TTG ACA GCT TAT CAT-3'.
```

Example 10

Construction of plasmids pINT73d, pINT88d and pINT89d

Plasmid pINT73d is a derivative of plasmid pINT69d (Example 8), in which the insulin precursor gene is arranged two times in succession. The plasmid therefore results in the formation of a polycistronic mRNA, which can double the yield. For its construction, a PCR reaction is carried out with plasmid pINT69d and the two oligonucleotides

```
Insu29:   5'-CTA GTA CTC GAG TTC AC-3'

          and
Insu11:   5'-TCA TGT TTG ACA GCT TAT CAT-3'.
```

This gives rise to a fragment with the insulin precursor gene and the pertinent ribosome binding site which in its 5'-end region has a cleavage site for enzyme XhoI and in its 3'-end region a cleavage site for SalI. The fragment is cut with the two above-mentioned enzymes and purified. Plasmid pINT69d is then linearized with SalI, the two DNA ends

produced are dephosphorylated with phosphatase (from calf intestine) and ligated with the fragment from the PCR reaction to yield plasmid pINT73d.

In an analogous manner there are obtained plasmids pINT88d and pINT89d when plasmid pINT72d (Example 9) is modified analogously by arranging the "miniproinsulin gene" twice or thrice in sequence.

Example 11

Construction of plasmid pINL41d

The starting plasmid pRUD3 has a structure analogous to that of plasmid pGATTP. However, instead of the trp-promoter region, it contains a tac-promoter region which is flanked by cleavage sites for enzymes EcoRI and Nco. The plasmid is cut with ECORI, whereupon the protruding ends of the cleavage site are filled in with Klenow polymerase. Cutting is performed subsequently with Nco and the ensuing promoter fragment is isolated.

The trp-promoter of plasmid pINT4Id is flanked by cleavage sites for enzymes PvuII and Nco. Since the plasmid has an additional cleavage site for PvuII, it is completely cut with Nco, but only partially with PvuII. The vector fragment, which is missing only the promoter region, is then isolated from the ensuing fragments. This is then ligated with the tac-promoter fragment to yield plasmid pINL4Id.

Example 12

Construction of plasmid pL4Ic

Plasmid pPL-lambda (which can be obtained from Pharmacia) has a lambda-pL-promoter region. The latter is flanked by nucleotide sequences:

5'GATCTCTCACCTACCAAACAAT3'

and

5'AGCTAACTGACAGGAGAATCC3'.

Oligonucleotides

LPL3: 5' ATGAATTCGATCTCTCACCTACCAAACAAT 3'

and

LPL4: 5' TTGCCATGGGGATTCTCCTGTCAGTTAGCT 3'

are prepared for additional flanking of the promoter region with cleavage sites for enzymes EcoRI and Nco. A PCR is carried out with these oligonucleotides and pPL-lambda and the resulting promoter fragment is cut with EcoRI and Nco and isolated. Plasmid pINL4Id is then also cut with these two enzymes and the ensuing vector fragment, which has no promoter, is then ligated with the lambda-pL-promoter fragment to yield plasmid pL41c.

Example 13

Construction of plasmid pL4Id

The trp-transcription terminator located between the resistance gene and the fusion protein gene in plasmid pL4Ic is not effective in E. coli strains that are suitable for fermentation (e.g. E. coli N4830-1). For this reason, a polycistronic mRNA and with it a large quantity of resistance gene product are formed in fermentation. To prevent this side reaction, the trp-terminator sequence is replaced by an effective terminator sequence of the E. coli-rrnB-operon. Plasmid pANG-MA has a structure similar to that of plasmid pINT41d, but it has an angiogenin gene instead of the fusion protein gene and an rrnB-terminator sequence (from commercial plasmid pKK223-3, which can be obtained from Pharmacia) instead of the trp-terminator sequence. The plasmid is cut with PvuI and SalI and the fragment containing the rrnB-terminator is isolated. Plasmid pL4Ic is then also cut with thes two enzymes and the fragment containing the insulin gene is

isolated. The two isolated fragments are then ligated to yield plasmid pL41d.

Example 14

Construction of plasmid pINTLI

To prepare a plasmid for general use in the expression of fusion proteins, the proinsulin gene of plasmid pINT41d is replaced by a polylinker sequence. This gene is flanked by cleavage sites for enzymes MluI and SalI. The plasmid is therefore cut with the help of the two above-mentioned enzymes and the vector fragment is isolated. This is then ligated, to yield plasmid pINTLI, with the following two synthetic oligonucleotides

```
            BstEII       AccI      EcoRI        KpnI     BamHI
5' CGCGCCTGGTTACCTCGAGGTATACTACGAATTCGAGCTCGGTACCCGGGGATCC
3'     GGACCAATGGAGCTCCATATGATGCTTAAGCTCGAGCCATGGGCCCCTAGG
                   XhoI                 SacI       XmaI


       SphI        XbaI
CTGCAGGCATGCAAGCTTGTCTAGAC    -3'
GACGTCCGTACGTTCGAACAGATCTGAGCT-5'
PstI        HindIII      (SalI).
```

Example 15

Insertion of a gene coding for HMG CoA-reductase (active domain) in pINTLI and expression of the fusion protein

Table 3 represents the DNA and amino acid sequence of the gene HMG CoA-reductase. The synthetic gene for HMG CoA-reductase known from EP-A O 292 803 (herein incorporated by reference) contains a cleavage site for BstEII in the region of amino acids Leu and Val in positions 3 and 4 (see Table 3). A protruding sequence corresponding to enzyme XbaI occurs at the end of the gene (in the noncoding area). The corresponding cleavage sites in the polylinker of plasmid pINTLI are in the same reading frame. Both cleavage sites are in each case singular.

Plasmid pUH10 contains the complete HMG gene (HMG fragments I, II, III, and IV), corresponding to the DNA sequence of table 3. Construction of pUH10 (figure 2) is described in EP-A 0 292 803 herein incorporated by reference. Briefly, special plasmids are prepared for the subcloning of the gene fragments HMG I to HMG IV and for the construction of the complete gene. These plasmids are derived from the commercially available vectors pUC18, pUC19 and M13mp18 or M13mp19, with the polylinker region having been replaced by a new synthetic polylinker corresponding to DNA sequence VI

```
                Nco          EcoRI     HindIII   BamHI    XbaI

                                          VI-1a
5'          AAT TGC CAT GGG CAT GCG GAA TTC CAA GCT TTG GAT CCA TCT AGA GGG
3'              CG GTA CCC GTA CGC CTT AAG GTT CGA AAC CTA GGT AGA TCT CCC TCG A
                                          VI-1b
```

These new plasmids have the advantage that, in contrast to the pUC and M13mp plasmids, they allow the cloning of DNA fragments having the protruding sequences for the restriction enzyme Nco. Moreover, the recognition sequences for the cleavage sites Nco, EcoRI, HindIII, BamHI, and XbaI are contained in the vectors in exactly the sequence in which they are present in the complete gene HMG, which facilitates the sequential cloning and the construction of this gene. Thus it is possible to subclone the gene fragments HMG I to HMG IV in the novel plasmids. After the gene fragments have been amplified, it is possible for the latter to be combined to give the complete gene (see below).

a. Preparation of vectors which contain DNA sequence VI

DNA sequence VI may be prepared by standard techniques. The commercially available plasmid pUC18 (or pUC19, M13mp18 or M13mp19) is opened with the restriction enzymes EcoRI/HindIII as stated by the manufacturer. The digestion mixture is fractionated by electrophoresis on a 1% agarose gel. The plasmid bands which have been visualized by ethidium bromide staining are cut out and eluted from the agarose by electrophoresis. 20 fmol of the residual plasmid thus obtained are then ligated with 200 fmol of the DNA fragment corresponding to DNA sequence VI at room temperature overnight. A new cloning vector pSU18 (or pSU19, M13mUS18 or M13mUS19) is obtained. In contrast to the commercially available starting plasmids, the new plamids can be cut with the restriction enzyme Nco. The restriction enzymes EcoRI and HindIII likewise cut the plasmids only once because the polylinker which is inserted via the EcoRI and HindIII cleavage sites destroys these cleavage sites which are originally present.

b. Preparation of the hybrid plasmids which contain the gene fragments HMG I to HMG IV.

i) Plasmid containing the gene fragment HMG I

The plasmid pSU18 is cut open with the restriction enzymes EcoRI and Nco in analogy to the description in Example 15 (a) above, and is ligated with the gene fragment I which has previously been phosphorylated.

ii) Plasmid containing the gene fragment HMG II

The plasmids with the gene subfragments HMG II-1, II-2 and II-3 are subjected to restriction enzyme digestion with EcoRI/MluI, MluI/BssHII or BssHII/HindIII to isolate the gene fragments HMG II-1, HMG II-2 or HMG II-3, respectively. The latter are then ligated in a known manner into the plasmid pSU18 which has been opened with EcoRI/HindIII.

iii) Plasmid containing the gene fragment HMG III

The plasmids with the gene subfragments HMG III-1 and III-3 are digested with the restriction enzymes EcoRI/HindIII and then cut with Sau96I to isolate the gene fragment HMG III-1, or with BamHI/BanII to isolate the gene fragment HMG III-3. These fragments can be inserted with the HMG III-2 fragment into a pSU18 plasmid which has been opened with HindIII/BamHI.

iv) Plasmid containing the gene fragment HMG IV

The plasmids with the gene subfragments HMG IV(1+2) and IV-(3+4) are opened with the restriction enzymes EcoRI/BamHI and EcoRI/XbaI, respectively, and the gene fragments HMG IV-(1+2) and HMG IV-(3+4) are purified by electrophoresis. The resulting fragments are then ligated into a pSU18 plasmid which has been opened with BamHI/XbaI and in which the EcoRI cleavage site has previously been destroyed with S1 nuclease as described below. A hybrid plasmid which still contains an additional AATT nucleotide sequence in the DNA sequence IV is obtained. The hybrid plasmid is opened at this point by digestion with the restriction enzyme EcoRI, and the protruding AATT ends are removed with S1 nuclease. For this purpose, 1 µg of plasmid is, after EcoRI digestion, incubated with 2 units of S1 nuclease in 50 mM sodium acetate buffer (pH 4.5), containing 200 mM NaCl and 1 mM zinc chloride, at 20°C for 30 minutes. The plasmid is recircularized in a known manner via the blunt ends. A hybrid plasmid which contains the gene fragment IV is obtained.

c. Construction of the hybrid plasmid pUH10 which contains the DNA sequence V

The hybrid plasmid with the gene fragment HMG I is opened with EcoRI/HindIII and ligated with the fragment HMG II which is obtained by restriction enzyme digestion of the corresponding hybrid plasmid with EcoRI/HindIII. The resulting plasmid is then opened with HindIII/BamHI and ligated with the fragment HMG III which can be obtained from the corresponding plasmid using HindIII/BamHI. The plasmid obtained in this way is in turn opened with BamHI/XbaI and linked to the fragment HMG IV which is obtained by digestion of the corresponding plasmid with BamHI/XbaI. The hybrid plasmid pUH10 which contains the complete HMG gene, corresponding to DNA sequence V, is obtained. Figure 2 shows the map of pUH10 diagrammatically, with "ori" and "Ap^r" indicating the orientation in the residual plasmid corresponding to pUC18.

If pINTLI is cut with BstEII and XbaI and the large fragment is isolated, and if, on the other hand, plasmid pUH10 (figure 2) is digested with the same enzymes and the fragment which encompasses most of the DNA sequenc V from this plasmid is isolated, after ligation of the two fragments we obtain a plasmid which codes a fusion protein in which

arginine follows the first eight amino acids in the ballast sequence of pINT41d (Table 1), which is followed, starting with Leu^3, by the structural gene of the active domain of HMG CoA-reductase. For purposes of comparison, the two initial plasmids are cut with enzymes Nco and XbaI and the corresponding fragments are ligated together, yielding a plasmid which codes, immediately after the start codon, the active domain of HMG CoA-reductase (in accordance with DNA sequence V of EP-A 0 292 803, see table 3).

Expression of the coded proteins occurs according to Example 4. Following the breakup of the cells, centrifugation is performed whereupon the expected protein of approximately 55 kDa is determined in the supernatant by gel electrophoresis. The band for the fusion protein is much more intensive here than for the protein expressed directly. Individual portions of 100 µl of the supernatant are tested in undiluted form, in a dilution of 1:10 and in a dilution of 1:100 for the formation of mevalonate. As an additional comparison, the fusion protein according to Example 4 (fusion protein with proinsulin constituent) is tested; no activity is apparent in any of the three concentrations. The fusion protein with the HMG CoA-reductase constituent exhibits maximum activity in all three dilutions, while the product of the direct expression shows graduated activity governed by the concentration. This indicates better expression of the fusion protein by a factor of at least 100.

Table 3 DNA sequence V + amino acid sequence I

NcoI 10 20

MET VAL LEU VAL THR GLN GLU PRO GLU ILE GLU LEU PRO ARG GLU PRO ARG PRO ASN GLU GLU CYS LEU GLN ILE LEU GLY ASN ALA GLU

5' CATG GTT CTG GTT ACC CAG GAA CCT GAA ATT GAA CTT CCG CGG GAA CCT CGG CCT AAT GAA GAA TGT CTA CAG ATC CTT GGT AAT GCA GAG

30 40 50

LYS GLY ALA LYS PHE LEU SER ASP ALA GLU ILE ILE GLN LEU VAL ASN ALA LYS HIS ILE PRO ALA TYR LYS LEU GLU THR LEU MET GLU

AAA GGT GCA AAG TTC CTT AGT GAC GCC GAG ATC ATC CAG TTA GTG AAT GCT AAA CAT ATC CCA GCT TAC AAG CTC GAG ACT CTG ATG GAA

60 70 80

THR HIS GLU ARG GLY VAL SER ILE ARG ARG GLN LEU LEU SER LYS LYS LEU SER GLU PRO SER SER LEU GLN TYR LEU PRO TYR ARG ASP

ACT CAT GAG CGT GGT GTA TCG ATT CGC CGA CAG TTA CTT TCG AAG AAA CTT TCT GAA CCG AGC TCT CTC CAG TAT CTG CCT TAT CGC GAT

90 100 110

TYR ASN TYR SER LEU VAL MET GLY ALA CYS CYS GLU ASN VAL ILE GLY TYR MET PRO ILE PRO VAL GLY VAL ALA GLY PRO LEU CYS LEU

TAT AAT TAC TCT TTG GTG ATG GGA GCC TGT TGT GAG AAT GTT ATT GGA TAT ATG CCC ATC CCT GTG GGA GTG GCA GGA CCT CTT TGC TTG

120 130 140

ASP GLU LYS GLU PHE GLN VAL PRO MET ALA THR THR GLU GLY CYS LEU VAL ALA SER THR ASN ARG GLY CYS ARG ALA ILE GLY LEU GLY

GAT GAA AAA GAA TTC CAG GTT CCA ATG GCA ACA ACA GAA GGT TGT CTT GTG GCT AGC ACC AAT AGA GGT TGC AGA GCG ATC GGT CTT GGT

150 160 170

GLY GLY ALA SER SER ARG VAL LEU ALA ASP GLY MET THR ARG GLY PRO VAL VAL ARG LEU PRO ARG ALA CYS ASP SER ALA GLU VAL LYS

GGA GGT GCC AGC TCC CGA GTT CTT GCA GAT GGT ATG ACG CGT GGC CCA GTT GTG CGT CTT CCA AGG GCT TGT GAC TCT GCA GAA GTG AAA

180 190 200

ALA TRP LEU GLU THR SER GLU GLY PHE ALA VAL ILE LYS GLU ALA PHE ASP SER THR SER ARG PHE ALA ARG LEU GLN LYS LEU HIS THR

GCA TGG CTC GAA ACA TCC GAA GGG TTC GCA GTG ATC AAG GAG GCA TTT GAC AGC ACT AGC AGA TTT GCG CGC CTT CAG AAA CTT CAT ACT

```
210                                 220                                 230
SER ILE ALA GLY ARG ASN LEU TYR ILE ARG PHE GLN SER ARG SER GLY ASP ALA MET GLY MET ASN MET ILE SER LYS GLY THR GLU LYS
AGT ATC GCT GGA CGC AAC CTT TAT ATC CGT TTC CAG TCC AGA TCT GGT GAC GCA ATG GGT ATG AAC ATG ATA TCT AAG GGC ACA GAG AAA

240                                 250                                 260
ALA LEU SER LYS LEU HIS GLU TYR PHE PRO GLU MET GLN ILE LEU ALA VAL SER GLY ASN TYR CYS THR ASP LYS LYS PRO ALA ALA ILE
GCA CTT TCC AAG CTT CAC GAG TAT TTT CCG GAA ATG CAG ATT CTG GCT GTT AGT GGT AAC TAT TGT ACT GAC AAG AAA CCT GCT GCT ATC

270                                 280                                 290
ASN TRP ILE GLU GLY ARG GLY LYS SER VAL VAL CYS GLU ALA VAL ILE PRO SER LYS VAL VAL ARG GLU VAL LEU LYS THR THR THR GLU
AAT TGG ATT GAG GGC CGT GGA AAA TCT GTT GTT TGT GAA GCT GTA ATT CCA AGC AAG GTT GTT AGA GAA GTT TTA AAG ACT ACC ACA GAG
```

```
300                                 310                                 320
ALA MET ILE GLU VAL ASN ILE ASN LYS ASN LEU VAL GLY SER ALA MET ALA GLY SER ILE GLY GLY TYR ASN ALA HIS ALA ALA ASN ILE
GCT ATG ATT GAG GTT AAT ATT AAC AAG AAT TTA GTG GGC TCT GCA ATG GCT GGT AGC ATC GGA GGC TAC AAC GCT CAT GCT GCA AAC ATT

330                                 340                                 350
VAL THR ALA ILE TYR ILE ALA CYS GLY GLN ASP ALA ALA GLN ASN VAL GLY SER SER ASN CYS ILE THR LEU MET GLU ALA SER GLY PRO
GTC ACC GCT ATC TAC ATT GCT TGT GGC CAG GAC GCA GCT CAG AAT GTT GGA TCC TCT AAC TGT ATT ACT TTA ATG GAA GCC TCA GGT CCG

360                                 370                                 380
THR ASN GLU ASP LEU TYR ILE SER CYS THR MET PRO SER ILE GLU ILE GLY THR VAL GLY GLY GLY THR ASN LEU LEU PRO GLN GLN ALA
ACA AAT GAA GAT TTA TAT ATC AGC TGC ACC ATG CCA TCT ATC GAG ATT GGT ACC GTG GGT GGT GGC ACC AAC CTT CTT CCA CAG CAG GCC

390                                 400                                 410
CYS LEU GLN MET LEU GLY VAL GLN GLY ALA CYS LYS ASP ASN PRO GLY GLU ASN ALA ARG GLN LEU ALA ARG ILE VAL CYS GLY THR VAL
TGT CTG CAA ATG TTG GGT GTT CAA GGA GCA TGC AAA GAT AAT CCT GGC GAA AAT GCC CGG CAA CTT GCA CGA ATT GTG TGT GGG ACC GTA
```

```
420                                                   430                                                  440
MET ALA GLY GLU LEU SER LEU MET ALA ALA LEU ALA ALA GLY HIS LEU VAL LYS SER HIS MET ILE HIS ASN ARG SER LYS ILE ASN LEU
ATG GCC GGC GAA TTG TCT CTT ATG GCT GCC TTG GCG GCC GGA CAT CTT GTC AAA TCT CAT ATG ATT CAC AAC CGT TCG AAG ATC AAT TTA

450                                                   460                 XbaI
GLN ASP LEU GLN GLY ALA CYS THR LYS LYS THR ALA --- ---
CAG GAT CTG CAA GGC GCT TGC ACC AAG AAG ACA GCA TAA TAG T
```

Example 16

Construction of plasmid pB70

Plasmid pINT41d is split with MluI and SalI and the large fragment is isolated. Plasmid pIK4 shown in figure 3a contains a gene for "mini-proinsulin," the C chain of which consists of arginine only.

The construction of this plasmid has previously been described in EP-A 0,347,781 (herein incorporated by reference). Briefly, the commercial plasmid pUC19 is opened using the restriction enzymes KpnI and PstI and the large fragment (figure 3-(1)) is separated through a 0.8% strength "Seaplaque" gel. This fragment is reacted with T4 DNA ligase using the DNA (figure 3-(2)) synthesized according to Table 4. Table 4 shows the sequence of gene fragment IK I, while table 5 represents the sequence of gene fragment IK II.

This ligation mixture then is incubated with competent E. coli 79/02 cells. The transformation mixture is plated out on IPTG/Xgal plates which contain 20 mg/l of ampicillin. The plasmid DNA is isolated from the white colonies and characterized by restriction and DNA sequence analysis. The desired plasmids are called pIK1 (figure 3).

Accordingly, the DNA (figure 3-(5)) according to Table 5 is ligated into pUC19 which has been opened using PstI and HindIII (figure 3-(4)). The plasmid pIK2 (figure 3) is obtained.

The DNA sequences (2) and (5) of figure 3 according to Table 4 and 5 are reisolated from the plasmids pIK1 and pIK2 and ligated with pUC19, which has been opened using KpnI and HindIII (figure 3-(7)). The plasmid pIK3 (figure 3) is thus obtained which encodes for a modified human insulin sequence.

The plasmid pIK3 is opened using MluI and SpeI and the large fragment (figure 3a-(9)) is isolated. This is ligated with the DNA sequence (10)

```
    B30        A1  A2  A3  A4  A5  A6  A7   A8    A9
    (Thr)(Arg) Gly Ile Val Glu Gln Cys Cys (Thr) (Ser)  (10)
5'    CG  CGT  GGT ATC GTT GAA CAA TGT TGT  A            3'
3'        A    CCA TAG CAA CTT GTT ACA ACA  TGA   TC     5'
    (MluI)                                        (SpeI)
```

which supplements the last codon of the B chain (B30) by one arginine codon and replaces the excised codon for the first 7 amino acids of the A chain and supplements the codon for the amino acids 8 and 9 of this chain. The plasmid pIK4 (figure 3a) is thus obtained which encodes for human mini-proinsulin.

In tables 4 and 5, the B- and A-chains of the insulin molecule are in each case indicated by the first and last amino acid. Next to the coding region in gene fragment IK II, there is a cleavage site for SalI which will be utilized in the following construction.

Plasmid pIK4 is cut with HpaI and SalI and the gene coding "mini-proinsulin" is isolated. This gene is ligated with the above-mentioned large fragment of pINT41d and the following synthetic DNA sequence.

```
                        B1
(Thr) Arg  Met  Gly  Arg  Phe
  CG  CGT  ATG  GGC  CGT  TTC  GTT
        A  TAC  CCG  GCA  AAG  CAA
(MluI)                        (HpaI)
```

This gives rise to plasmid pB70, which codes a fusion protein in which the ballast sequence (Table 1, line 1) is followed by amino acid sequence Met-Gly-Arg which is followed by the amino acid sequence of the "mini-proinsulin".

TABLE 4: Gene fragment IK I (2)

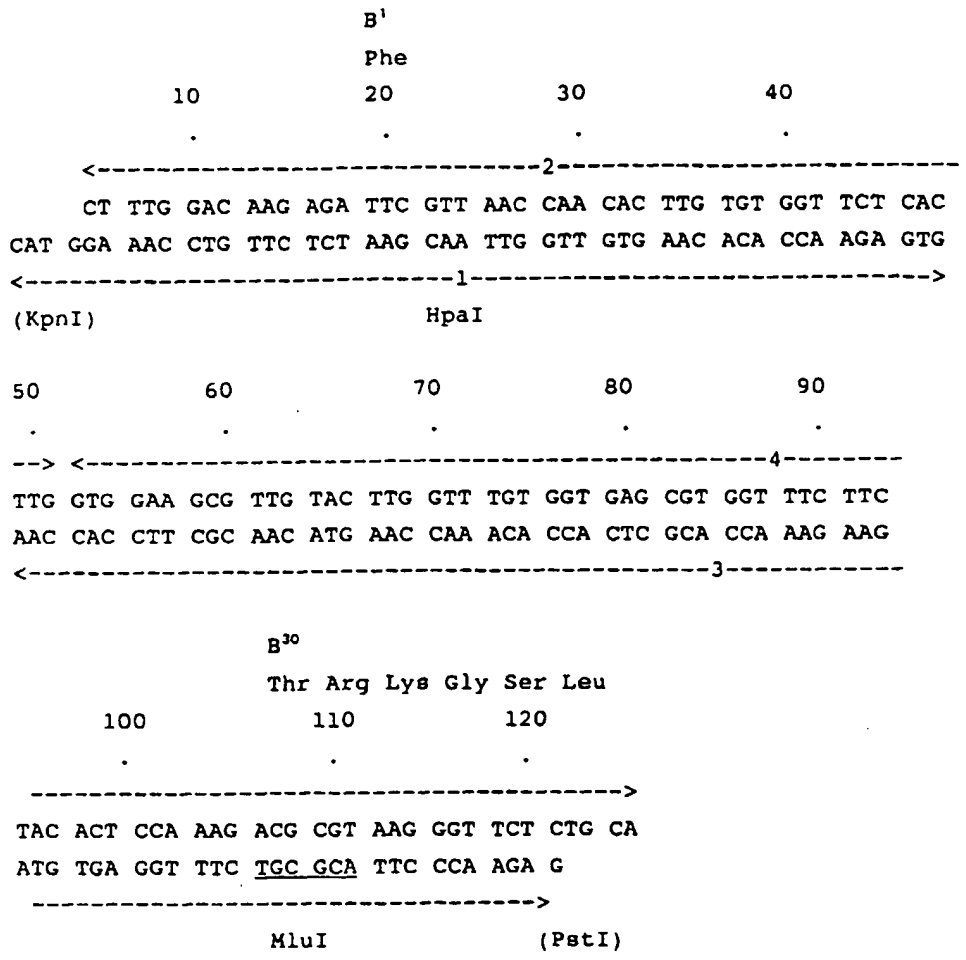
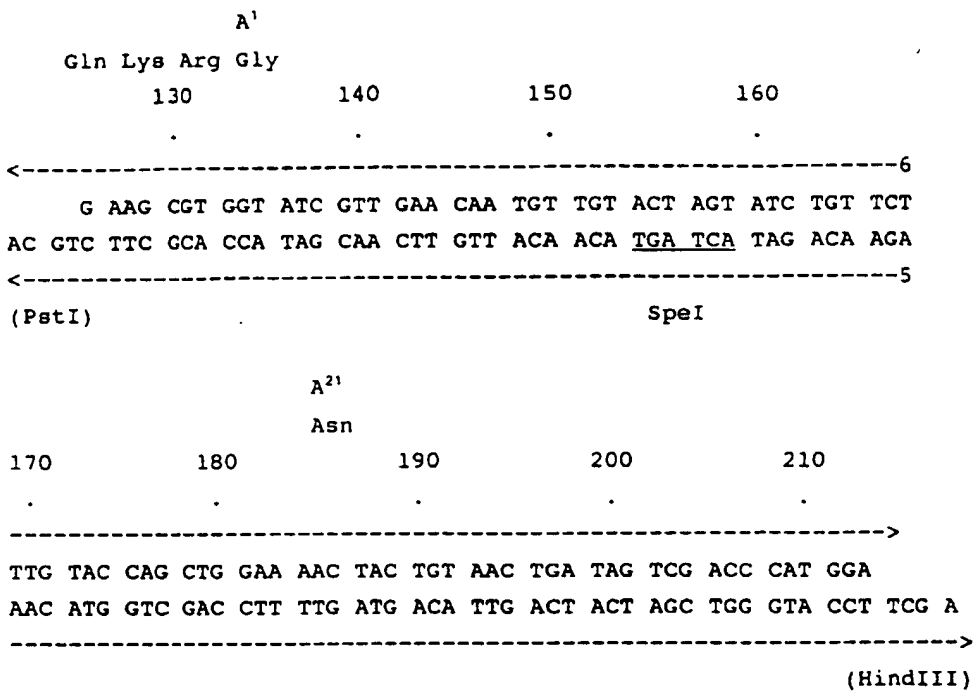


Table 5: Gene fragment IK II (5)



Example 17

By using the oligonucleotides listed below there are obtained plasmids pINT90d to pINT96d in analogy to the previous examples. An asterisk indicates the same encoded amino acid in the ballast constituent as in pINT4ld.

pINT92 encodes a double mutation in the insulin derivative encoded by the plasmid pINT72d since the codon for Arg at the end of the ballast constituent and in the "mini C chain" is substituted by the codon for Met. Thus the expressed preproduct can be cleaved with cyanogen bromide.

pINT90d: ******GNSA* (variant of pINT69d)
TIR: 5'-CTGAAATGAGCTGTTGAC-3
and
Insu50: 5'-TGCCGAATTTCCTGTTGATGTTGTTGC-3'
or
Insu49: 5'-GGAAATTCGGCACGATTTGTGAACCAG-3'
and
Insu11: 5'-TCATGTTTGACAGCTTATCAT-3'

pINT91d: ******GNSA* (variant of pINT72d)
TIR: 5'-CTGAAATGAGCTGTTGAC-3'
and
Insu50: 5'-TGCCGAATTTCCTGTTGATGTTGTTGC-3'
or
Insu49: 5'-GGAAATTCGGCACGATTTGTGAACCAG-3'
and
Insu11: 5'-TCATGTTTGACAGCTTATCAT-3'

pINT92d: (double mutant of pINT72d)
Insu56: 5′-TCGACCATGGCAACAACATCAACAATGTTTGTG-3
and
Insu58: 5′-GATGCCCATGGTCTT-3′
or
Insu57: 5′-AAGACCATGGGCATC-3′
and
Insu11: 5′-TCATGTTTGACAGCTTATCAT-3′

pINT93d: ******(variant of pINT68d)
Insu53: 5′-ACCATGGCAACAACATCAACAAAACGATTTGTG-3′
and
Insu11: 5′-TCATGTTTGACAGCTTATCAT-3′

pINT94d: ******(variant of pINT68d)
Insu54: 5′-ACCATGGCAACAACATCAACACCACGATTTGTG-3′
and
Insu11: 5′-TCATGTTTGACAGCTTATCAT-3′

pINT95d: ******(variant of pINT68d)
Insu55: 5′-TCGACCATGGCAACAACATCAACAATGCGATTTGTG-3′
and
Insu11: 5′-TCATGTTTGACAGCTTATCAT-3′

pINT96d: ******(variant of pINT68d)
Insu71: 5′-ACCATGGCAACAACATCAACAGGACGATTTGTG-3′
and
Insu11: 5′-TCATGTTTGACAGCTTATCAT-3′

Claims

1. A process for the preparation pf fusion proteins, which fusion proteins contain a desired protein and a ballast constituent, which process comprises

(a) constructing a mixed oligonucleotide which codes for the said ballast constituent, wherein the said oligonucleotide contains the DNA sequence (coding strand)

$$(DCD)_x$$

in which D is A, G or T and x is 4 to 12,
(b) inserting the said mixed oligonucleotide into a vector so that it is functionally linked to a regulatory region and to the structural gene coding for the said desired protein,
(c) transforming host cells with the so-obtained vector population and
(d) selecting from the transformants one or more clones expressing a fusion protein in high yield.

2. The process as claimed in claim 1, wherein the said oligonucleodie codes at its 3' end of the coding strand for an amino acid or for a group of amino acids which allows an easy cleavage of the said desired protein from the said ballast constituent.

3. The process as claimed in claim 2, wherein said cleavage is an enzymatic cleavage.

4. The process as claimed in claim 1, wherein the said oligonucleotide is designed so that it leads to a fusion protein which is soluble or which easily can be solubilized.

5. The process as claimed in claim 1, wherein the said oligonucleotide is designed so that the ballast constituent does not interfere with folding of the said desired protein.

6. The process as claimed in claim 1, wherein x is 4 to 8.

7. The process as claimed in claim 5, wherein the said oligonucleotide has the sequence (coding strand)

$$ATG\ (DCD)_y\ (NNN)_z$$

wherein N in the NNN triplet stands for identical or different nucleotides, excluding stop codons for NNN, z is 1 to 4 and y + z is 6 to 12, y being at least 4.

8. The process as claimed in claim 7, wherein y + z is 6 to 10.

9. The process as claimed in claim 7, wherein y is 5 to 8 and z is 1.

10. The process as claimed in claim 1, wherein the said oligonucleotide has the sequence (coding strand)

$$ATG\ GCW\ (DCD)_{4\text{-}8}\ CGW$$

in which W is A or T.

Patentansprüche

1. Verfahren zur Herstellung von Fusionsproteinen, welche Fusionsproteine ein gewünschtes Protein und einen Ballastbestandteil enthalten, welches Verfahren umfaßt

(a) das Konstruieren eines gemischten Oligonucleotids, welches für den genannten Ballastbestandteil codiert, wobei das genannte Oligonucleotid die DNA-Sequenz (codierender Strang)

$$(DCD)_x$$

enthält, worin D für A, G oder T steht und x von 4 bis 12 beträgt;
(b) das Insertieren des genannten gemischten Oligonucleotids in einen Vektor, so daß dieses an eine regulatorische Region und an das Strukturgen, welches für das gewünschte Protein codiert, funktionell gebunden ist;
(c) das Transformieren der Wirtszellen mit der so erhaltenen Vektorpopulation; und
(d) das Selektieren von einem oder mehreren Klonen, welche ein Fusionsprotein in hoher Ausbeute exprimieren, aus den Transformanten.

2. Verfahren nach Anspruch 1, worin das genannte Oligonucleotid an seinem 3'-Ende des codierenden Stranges für eine Aminosäure oder eine Gruppe von Aminosäuren codiert, wodurch eine leichte Spaltung des gewünschten Proteins von dem genannten Ballastbestandteil ermöglicht wird.

3. Verfahren nach Anspruch 2, worin die genannte Spaltung eine enzymatische Spaltung ist.

4. Verfahren nach Anspruch 1, worin das genannte Oligonucleotid so ausgestaltet ist, daß es zu einem Fusionsprotein führt, welches löslich ist oder welches leicht solubilisiert werden kann.

5. Verfahren nach Anspruch 1, worin das genannte Oligonucleotid so ausgestaltet ist, daß der Ballastbestandteil die Faltung des genannten gewünschten Proteins nicht beeinträchtigt.

6. Verfahren nach Anspruch 1, worin x von 4 bis 8 beträgt.

7. Verfahren nach Anspruch 5, worin das genannte Oligonucleotid die Sequenz (codierender Strang)

$$ATG\,(DCD)_y\,(NNN)_z$$

besitzt, worin N im NNN-Triplett für identische oder verschiedene Nukleotide steht, wobei Stopcodons für NNN ausgeschlossen sind, z von 1 bis 4 beträgt und y+z von 6 bis 12 beträgt, wobei y mindestens 4 ist.

8. Verfahren nach Anspruch 7, worin y+z von 6 bis 10 beträgt.

9. Verfahren nach Anspruch 7, worin y von 5 bis 8 beträgt und z 1 ist.

10. Verfahren nach Anspruch 1, worin das genannte Oligonucleotid die Sequenz (codierender Strang)

$$ATG\ GCW\ (DCD)_{4-8}\ CGW$$

besitzt, worin W A oder T ist.

Revendications

1. Procédé pour la préparation de protéines de fusion, lesquelles protéines de fusion contiennent une protéine recherchée et un constituant de lestage, lequel procédé comprend

(a) la construction d'un oligonucléotide mixte codant pour ledit constituant de lestage, ledit oligonucléotide contenant la séquence d'ADN (brin codant)

$$(DCD)_x$$

dans laquelle D est A, G ou T et x va de 4 à 12,
(b) l'insertion dudit oligonucléotide mixt dans un vecteur, d manièr qu'il soit fonctionnellement lié à une

région régulatrice et au gène structural codant pour ladite protéine recherchée,
(c) la transformation de cellules hôtes par la population de vecteurs ainsi obtenue et
(d) la sélection, à partir des transformants, d'un ou plusieurs clones exprimant une protéine de fusion avec un rendement élevé.

2. Procédé selon la revendication 1, dans lequel ledit oligonucléotide code à son extrémité 3' du brin codant pour un aminoacide ou pour un groupe d'aminoacides permettant une séparation aisée de ladite protéine recherchée d'avec ledit constituant de lestage.

3. Procédé selon la revendication 2, dans lequel ladite séparation est une coupure enzymatique.

4. Procédé selon la revendication 1, dans lequel ledit oligonucléotide est conçu de manière à conduire à une protéine de fusion qui est soluble ou qui peut être aisément solubilisée.

5. Procédé selon la revendication 1, dans lequel ledit oligonucléotide est conçu de manière que le constituant de lestage n'interfère pas avec le repliement de ladite protéine recherchée.

6. Procédé selon la revendication 1, dans lequel x va de 4 à 8.

7. Procédé selon la revendication 5, dans lequel ledit oligonucléotide comporte la séquence (brin codant)

$$ATG\ (DCD)_y\ (NNN)_z$$

dans laquelle N dans le triplet NNN représente des nucléotides identiques ou différents, à l'exclusion des codons d'arrêt pour NNN, z va de 1 à 4 et y + z va de 6 à 12, y étant au moins égal à 4.

8. Procédé selon la revendication 7, dans lequel y + z va de 6 à 10.

9. Procédé selon la revendication 7, dans lequel y va de 5 à 8 et z est égal à 1.

10. Procédé selon la revendication 1, dans lequel ledit oligonucléotide comporte la séquence (brin codant)

$$ATG\ GCW\ (DCD)_{4-8}\ CGW$$

dans laquelle W est A ou T.

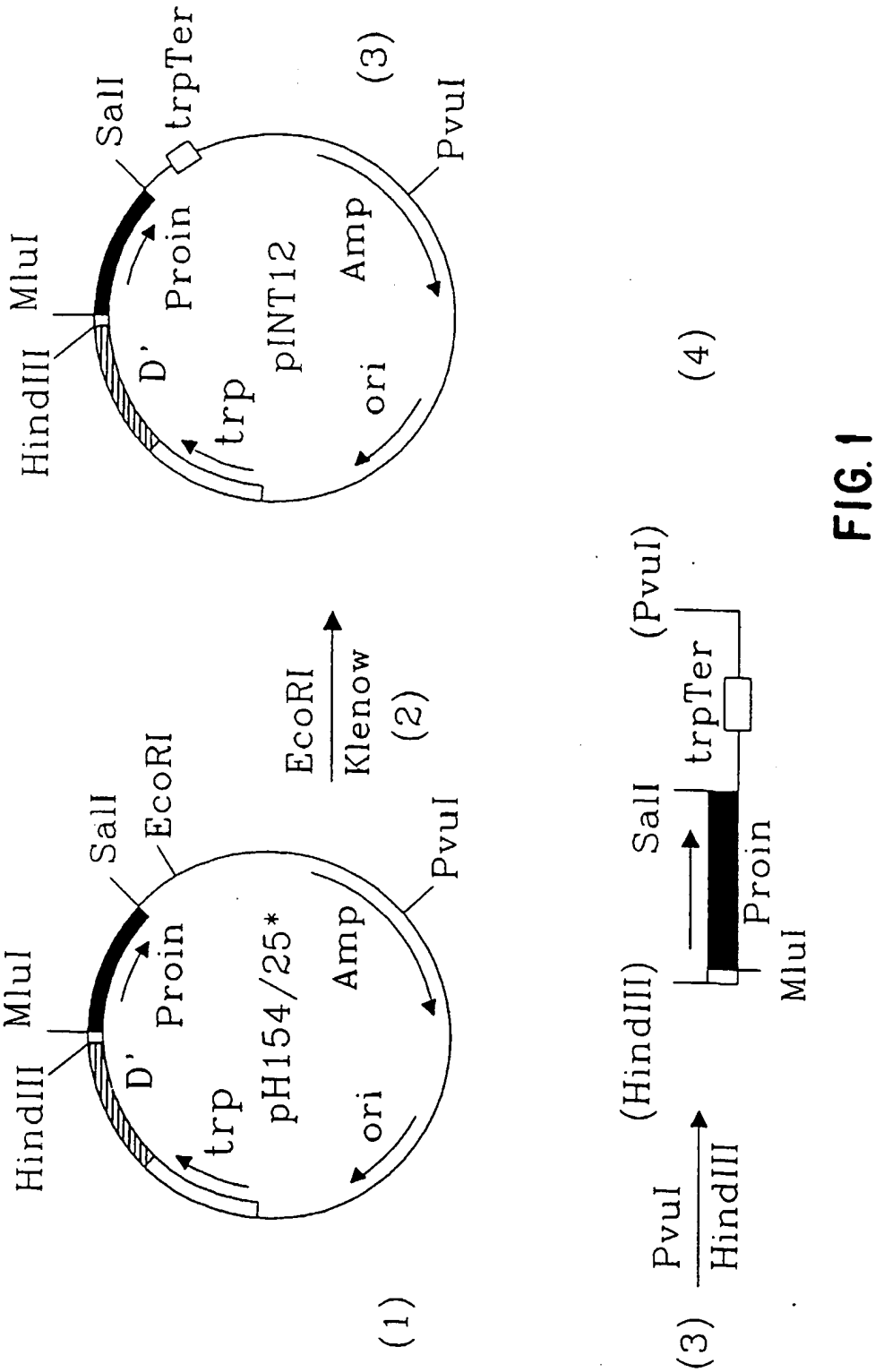

HindIII
MluI
SalI
EcoRI
PvuI
D'
Proin
trp
pH154/25*
Amp
ori
(1)
EcoRI
Klenow
(2)
HindIII
MluI
SalI
trpTer
PvuI
D'
Proin
trp
pINT12
Amp
ori
(3)
(3)
PvuI
HindIII
(HindIII)
SalI
trpTer
(PvuI)
Proin
MluI
(4)

FIG. 1

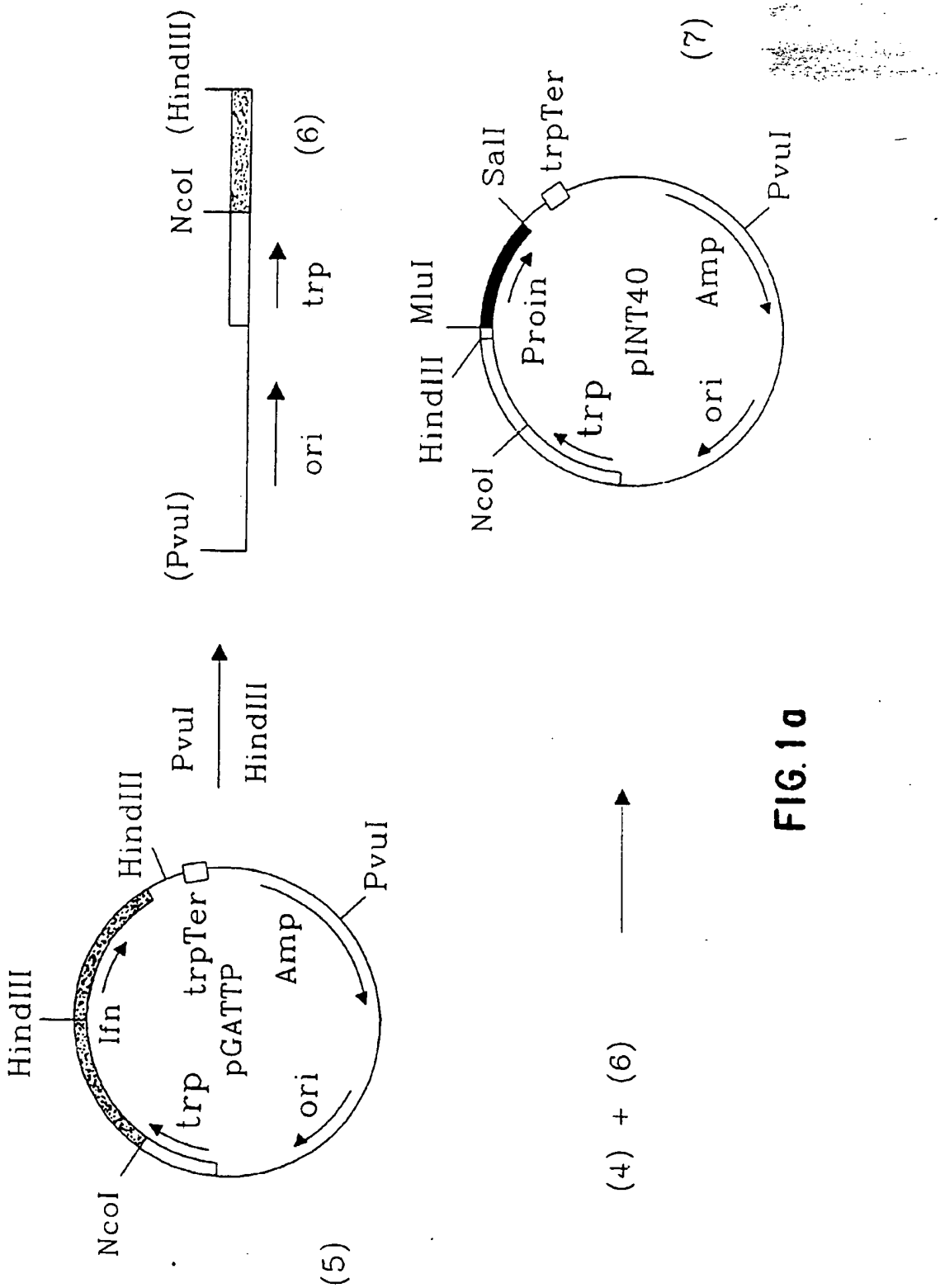

HindIII
Ifn
trp
trpTer
pGATTP
Amp
ori
NcoI
PvuI
HindIII
PvuI
HindIII
(5)
(4) + (6)
NcoI
(PvuI)
ori
trp
NcoI
(HindIII)
(6)
HindIII
MluI
Proin
trp
pINT40
Amp
ori
NcoI
SalI
trpTer
PvuI
(7)

FIG.1a

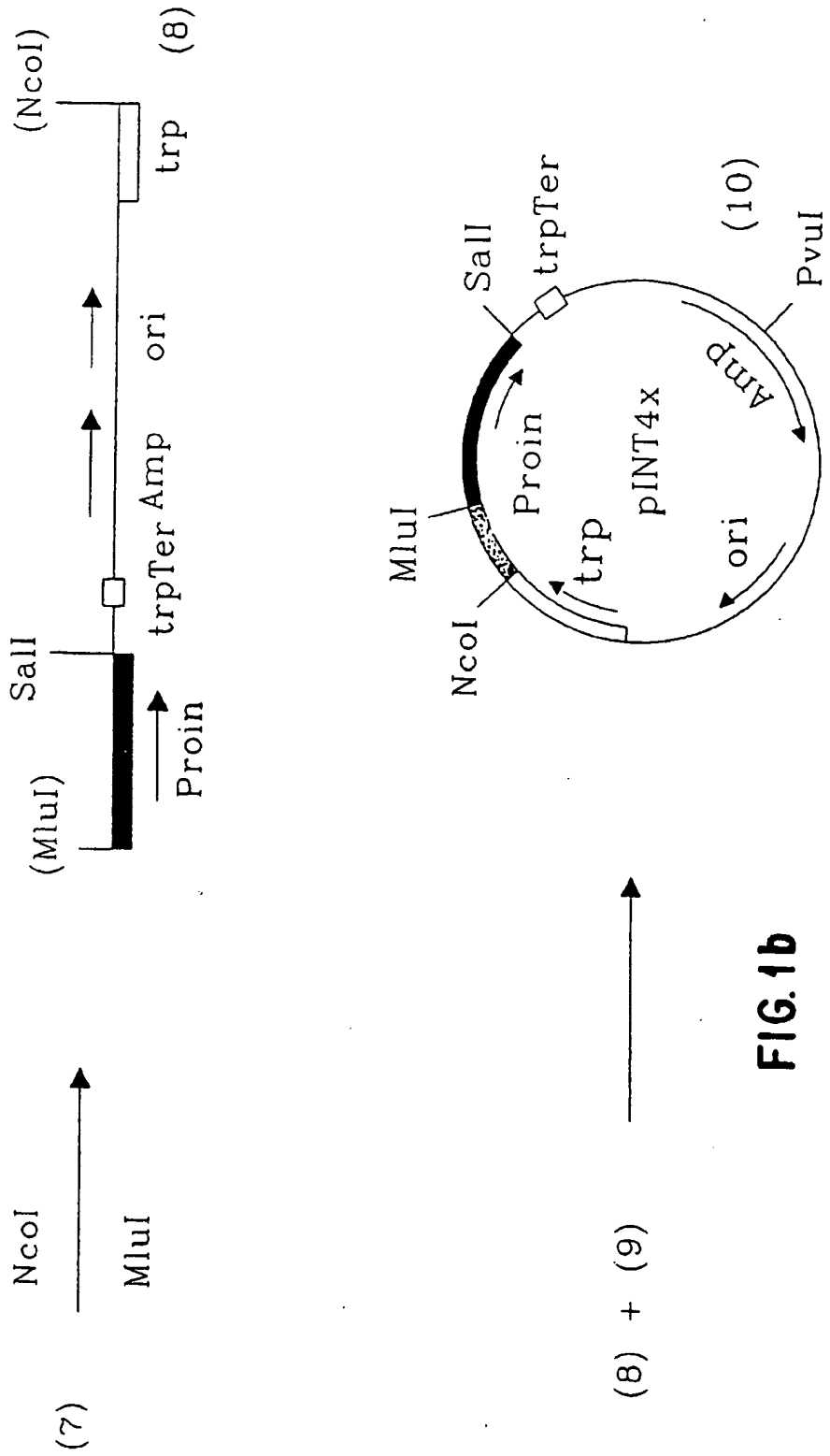

(NcoI)
trp
(8)
SalI
trpTer
Amp
ori
(MluI)
Proin
NcoI
MluI
(7)
(8) + (9)
SalI
trpTer
(10)
PvuI
MluI
Proin
pINT4x
Amp
trp
ori
NcoI

FIG.1b

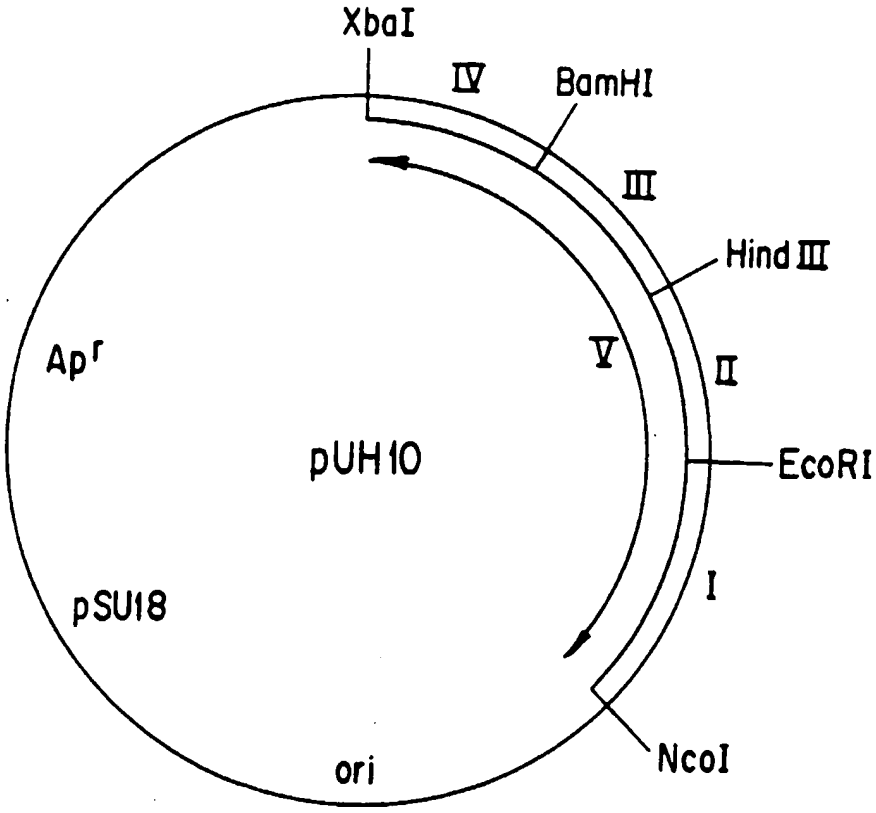

XbaI
IV
BamHI
III
Hind III
II
EcoRI
I
NcoI
ori
pSU18
Ap^r
V
pUH10

FIG. 2

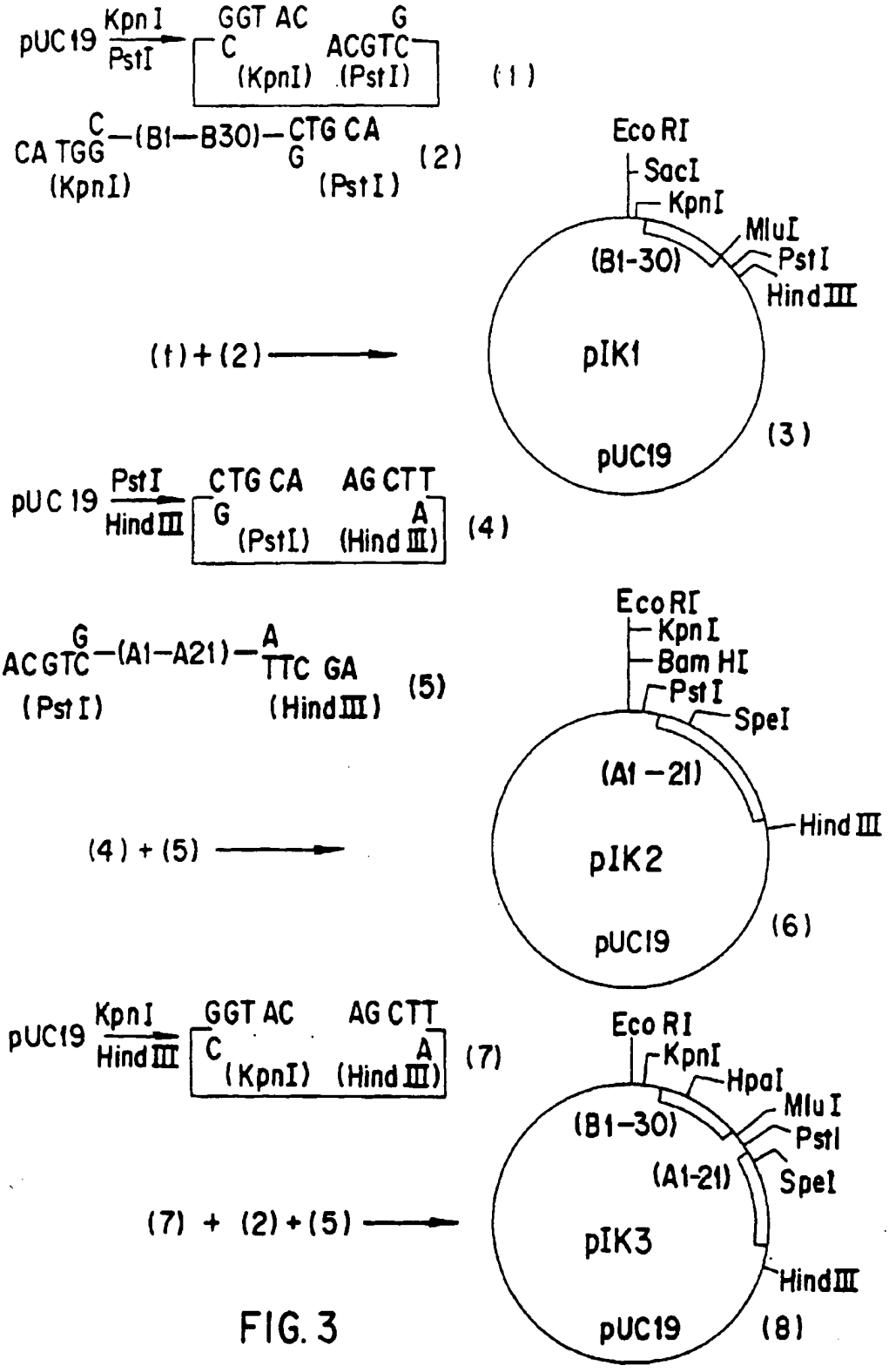

pUC19
KpnI
PstI
GGT AC
C
(KpnI)
G
ACGTC
(PstI)
(1)
C
CA TGG
(KpnI)
(B1—B30)
CTG CA
G
(PstI)
(2)
(1)+(2)
EcoRI
SacI
KpnI
MluI
PstI
HindIII
(B1-30)
pIK1
pUC19
(3)
pUC19
PstI
HindIII
CTG CA
G
(PstI)
AG CTT
A
(HindIII)
(4)
G
ACGTC
(PstI)
(A1—A21)
A
TTC GA
(HindIII)
(5)
(4)+(5)
EcoRI
KpnI
BamHI
PstI
SpeI
(A1—21)
HindIII
pIK2
pUC19
(6)
pUC19
KpnI
HindIII
GGT AC
C
(KpnI)
AG CTT
A
(HindIII)
(7)
(7) + (2)+(5)
EcoRI
KpnI
HpaI
MluI
(B1-30)
PstI
(A1-21)
SpeI
pIK3
HindIII
pUC19
(8)

FIG. 3

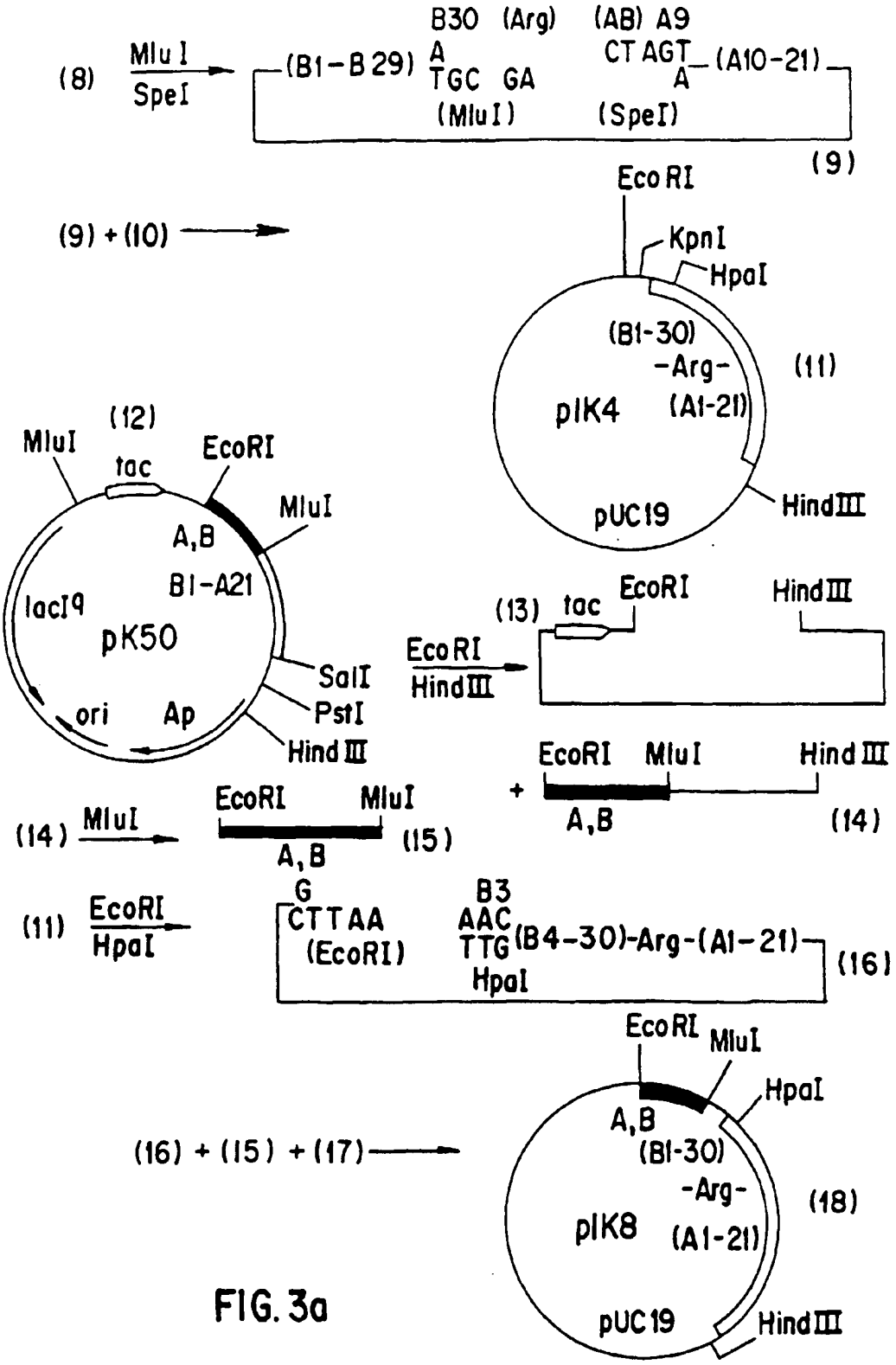

(8)
MluI
SpeI
(B1-B29)
B30 (Arg) (AB) A9
A
TGC GA
(MluI)
CT AGT
A
(A10-21)
(SpeI)
(9)
(9) + (10)
EcoRI
KpnI
HpaI
(B1-30)
-Arg-
(A1-21)
pIK4
(11)
pUC19
HindIII
(12)
MluI
tac
EcoRI
MluI
A,B
B1-A21
lacIq
pK50
SalI
PstI
ori
Ap
Hind III
(13)
tac
EcoRI
HindIII
EcoRI
HindIII
EcoRI
MluI
Hind III
+
A,B
(14)
(14)
MluI
EcoRI
MluI
A,B
(15)
(11)
EcoRI
HpaI
G
CTTAA
(EcoRI)
B3
AAC
TTG
(B4-30)-Arg-(A1-21)
HpaI
(16)
(16) + (15) + (17)
EcoRI
MluI
HpaI
A,B
(B1-30)
-Arg-
(A1-21)
pIK8
(18)
pUC19
HindIII

FIG. 3a